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# FRAXE Mutation Analysis in Three Spanish Families

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**Very little is known about the phenotype of FRAXE-positive individuals and the relation between the genotype/phenotype and genotype/cytogenetic expression. We describe three families with normal and mildly affected individuals and a severely retarded male expressing fragility at the FRAXE locus or presenting different expansions at the CGG FRAXE triplet. In addition, we analyze the FRAXE mutation in sperm DNA from a retarded male carrier with a handicapped daughter expressing fragility at the FRAXE locus. Mental status in FRAXE individuals is highly variable and, although mild mental retardation is observed in most cases, several carrier males are apparently normal. It seems that methylation is not as strictly associated with size of CGG triplets in the FRAXE locus as in FRAXA, and it is possible that normal carrier individuals with fully methylated increments in lymphocytes have a certain proportion of unmethylated alleles in the critical (i.e., neural) tissues. FRAXE mutation is apparently similar to FRAXA in that males with somatic large methylated increments are carriers of small unmethylated ones in germinal cells.** © 1996 Wiley-Liss, Inc.

**KEY WORDS: fragile site, FRAXE, X-linked mental retardation**

## INTRODUCTION

Three folate-sensitive fragile sites, FRAXA, FRAXE, and FRAXF, have been identified on the distal end of the long arm of the X chromosome (Xq), and these sites contain expanded, hypermethylated, and unstable CGG (or GCC) repeats near CpG islands.

The fragile site FRAXA is associated with the fragile X syndrome, the most common form of inherited mental retardation [for review, see Fryns, 1989], which is molecularly characterized by an unstable CGG repeat at the 5′ end of the FMR-1 gene [Verkerk et al., 1991]. Abnormal expansion of the repeat beyond approximately 230 copies results in the methylation of CpG residues and transcriptional silencing of the gene [Pieretti et al., 1991].

In some families ascertained by fragility in the distal Xq, in which FRAXA CGG amplification is absent, fluorescence in situ hybridization shows that the fragility lies distal to FRAXA and corresponds to FRAXE or FRAXF loci.

The FRAXE fragile site has been cloned [Knight et al., 1993], and individuals with cytogenetic expression have amplification of this CGG repeat. In normal individuals, 6–25 copies of the triplet are present, with an average of 15 copies. In individuals who express FRAXE, more than 200 copies of the triplet can be found. In these individuals, a CpG island proximal to the CGG repeat was methylated, suggesting that methylation could play a role similar to FRAXA in the inactivation of a putative gene in the FRAXE region.

Whereas FRAXF fragility does not contribute to an obvious phenotype in carrier males [Parrish et al., 1994], a relation between FRAXE and mild mental impairment is suggested by the occurrence of more mentally impaired male and female carriers, after removing index cases in affected families, than could be expected by chance [Mulley et al., 1995].

Very little is known about the phenotype of FRAXE-positive individuals and the relation between the genotype/phenotype and genotype/cytogenetic expression.

In general, reported males who did express the fragile site FRAXE were mildly retarded. But several individuals considered intellectually and physically normal expressed the FRAXE fragile site; amplification of the CGG repeat showed methylation across the FRAXE CpG island [Knight et al., 1993, 1994; Mulley et al., 1995]. In addition, a mildly retarded male, with a small methylated increment, did not express the FRAXE locus [Knight et al., 1994].

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The variable penetrance observed in previously reported FRAXE families, in which mental impairment is not apparently cosegregating with the expression of the fragile site or with the triplet increment, might be explained by the existence of somatic mosaicism with normal expression of the FRAXE gene product in the critical tissues [Knight et al., 1993].

The FRAXE expanded CGG repeat is unstable when passing through either the male or female line. Generally, it expands when passing through the female line, although some reductions have been seen. By contrast, carrier males generally transmit an unmethylated reduced fragment to their normal daughters, a situation similar to that seen in FRAXA, where only small expansions are present in the sperm of carriers males [Reyners et al., 1993]. However, a report of an affected male, apparently mosaic for FRAXE, who has had an affected nonexpressing daughter, has been published [Hamel et al., 1994].

We describe three families with normal and mildly affected individuals and a severely retarded male who expresses fragility at the FRAXE locus or presents different expansions at the CGG FRAXE triplets. In addition, we analyze the FRAXE mutation in sperm DNA from a retarded carrier male with a handicapped daughter who expresses fragility in the FRAXE locus.

## PATIENTS, MATERIAL, AND METHODS

### Family Data

The families described in this report (Fig. 1) were ascertained by the referral of a mentally impaired person for routine cytogenetic analysis, and this led to the detection of a fragile site in the distal Xq.

There were more mentally affected members in families 1 and 2 than in family 3. In families 2 and 3, cytogenetic prenatal diagnosis was done in two carrier women expressing fragility at Xq27-28. All the families were excluded as FRAXA and classified as FRAXE by direct Southern analysis with probes StB12.3 and OxE20. In family 1, segregation analyses of the fragile X chromosome was analyzed with 1A1 and U6.2 probes. Families 1 and 2 were reported as families 5 and 6 in Mulley et al. [1995].

In family 1 (Fig. 1a), the propositus (II-6) is a slightly retarded 13-year-old boy. His maternal cousin (II-9) is a moderately retarded 19-year-old boy. The patient's mother (I-1) is mildly retarded, and his aunt (I-3) has borderline intelligence.

In family 2 (Fig. 1b), the index case (III-1) is a 19-year-old severely retarded boy. His youngest brother (III-4), 13 years old, is mildly retarded and needs special schooling. Two brothers and 1 sister are apparently normal. The mother (II-2) is mildly retarded, and the aunt (II-3) is dull. Two uncles (II-7 and II-8) are slightly retarded, and the daughter of one (III-8) is 13 years old and mildly retarded. The other relatives are apparently normal.

In family 3 (Fig. 1c), the index case (II-3) is a 23-year-old mentally retarded man. His sister (II-1), 37 years old, is apparently normal. No other relatives are mentally retarded.

### Clinical Data in Families 2 and 3

***Family 2 (Fig* 1b).** The propositus (III-1) is a 19-year-old boy with severe mental retardation and hyperactive behavior. He has a brachycephalic skull with normal head circumference (56.5 cm), and craniofacial disproportion with a narrow and sloping forehead and broad midface. The supraorbital ridges are prominent. The occiput is flattened with low hairline in the broad neck and forehead.

The interpupilar distance is normal, but he has mild telecanthus and blepharophimosis. He has a broad nose and nares, with a wide nasal septum and normal philtrum, full lips, and high arched palate. The ears are not prominent and are normal in size and position, with posterior rotation and attached ear lobe.

Other anomalies are a narrow chest, with pectus excavatum of the lower sternum and pectus carinatus of the upper sternum, and mild kyphoscoliosis. The limbs shows wide hands with clinodactyly of the fourth and fifth fingers and normal extensibility of joints. Testes are normal. The stature is 170 cm.

Individual III-4 is a 13-year-old boy, who performs poorly at school but is not hyperactive. At physical examination, he shows brachycephaly with a normal head circumference (55 cm), broad neck, sloping forehead, prominent supraorbital ridges, flattened occiput with low hairline in the neck and forehead, broad nose and nasal bridge with anteverted nostrils and wide septum, left epicanthal fold, telecanthus and normal interpupillary distance, full lips, high palate, and slight micrognathia. The auricles are not prominent and are normal in size and position, with posterior rotation and attached ear lobe.

Other anomalies include pectus excavatum of the lower sternum, pectus carinatus of the upper sternum, and mild kyphoscoliosis. He has normal hands and extensibility of joints, cubitus valgus, and pes planus. His testes are normal.

***Family 3 (Fig* 1c).** The index case (II-3) is a 23-year-old man with severe mental retardation. He has brachycephalia and mild microcephaly with an abnormal head circumference (53 cm, 3rd centile), a narrow forehead, and flattened occiput. The auricles are normal, and he has a high arched palate. He has pectus excavatum and carinatus. Hands and extensibility of joints are normal. His testes are abnormal, with a volume of 50 ml in the left testis and 35 ml in the right testis.

### Cytogenetic Analysis

Duplicate peripheral blood cultures were set in Hepes-buffered 199 medium supplemented with 3% fetal calf serum. One culture induced with methotrexate (5 $\mu$g/ml) or FUdR 0.1 M during the final 24 hr. Fifty GTG-banded metaphases were examined from each culture.

### Molecular Analysis

Total DNA was extracted by standard methods from blood and sperm samples. Ten micrograms of DNA were digested from each case. The digested samples were run on 20-×-25-cm 0.8% agarose gels and allowed

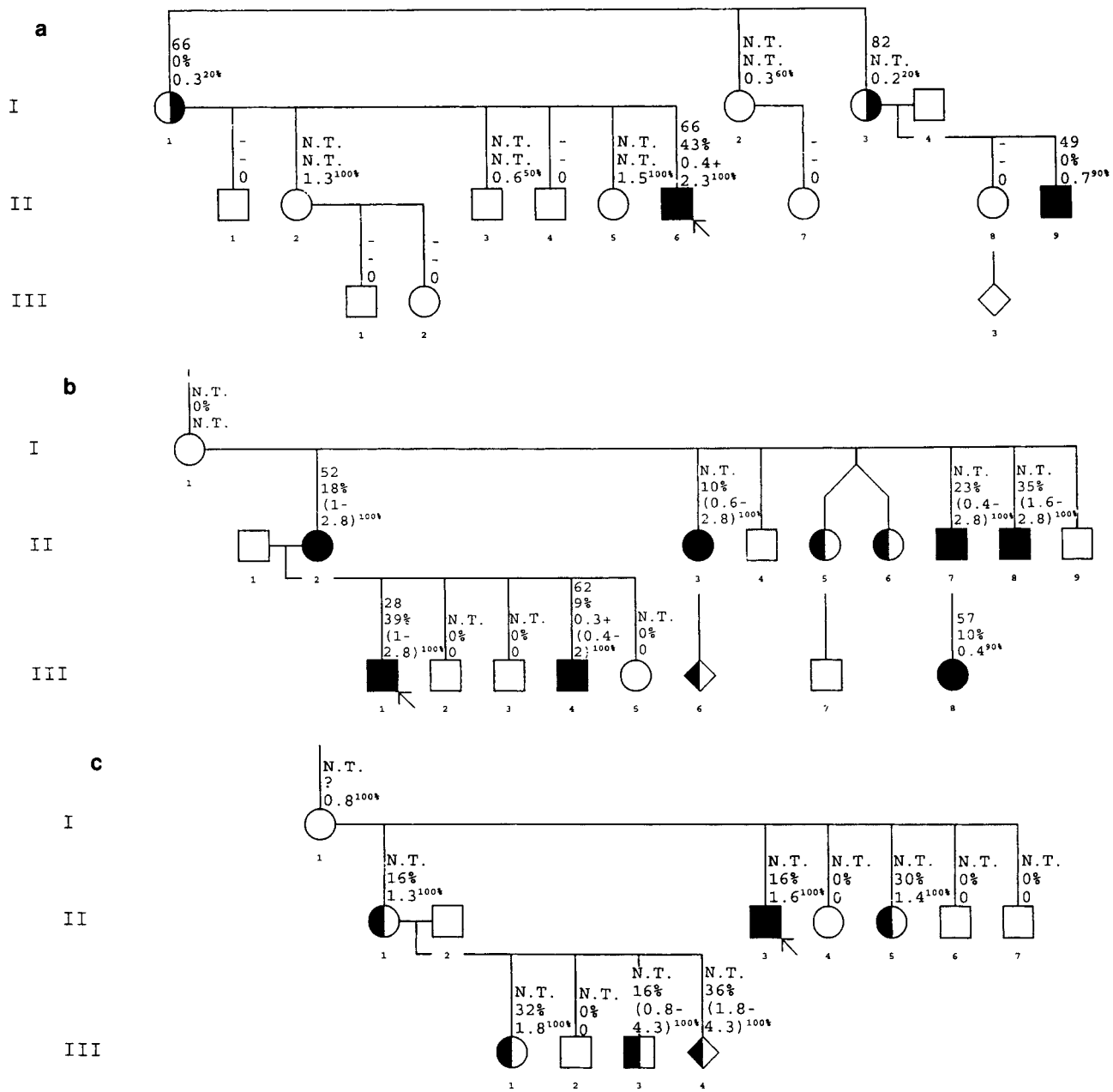


Fig. 1. Pedigrees of families 1 (**a**), 2 (**b**), and 3 (**c**), with IQ values, percentage of cytogenetic expression of fragile site, and increment (Δ, in Kb) for FRAXE locus. The plus sign indicates that more than 1 expanded fragment exists. The smear limits are shown in parentheses. The estimated degree of methylation as a percentage, for the FRAXE-associated CpG island, is given as a superscript to the FRAXE value. N.T. and the minus sign indicate untested values. Black/white squares and circles indicate FRAXE+ individuals, and white/black squares and circles indicate mentally impaired individuals.

to migrate at 65 V for 20 hr. Electrophoresed samples were transferred onto a Hybond-$N^+$ membrane (Amersham). FRAXA locus analysis was performed by hybridizing a $^{32}$P-labeled StB12.3 probe with EcoRI/EagI double-digested DNA samples. FRAXE locus analysis was performed by hybridizing a $^{32}$P-labeled OxE20 probe with DNA samples HindIII digested to measure size increment. Methylation status of the FRAXE-associated CpG islands was assessed by HindIII/NotI double digestion and estimated simply as the relative intensity of the expanded HindIII fragment and the expanded HindIII/NotI fragment.

Size increments are expressed as delta (Δ) over the normal fragment (active X chromosome of approximately 2.8 Kb and inactive X chromosome of approximately 5.2 Kb).

## Psychological Assessment

To determine the overall IQ of patients and relatives, the Weschler (WISC or WAIS) and Stanford-Binet (Terman-Merrill, L-M) scales were applied.

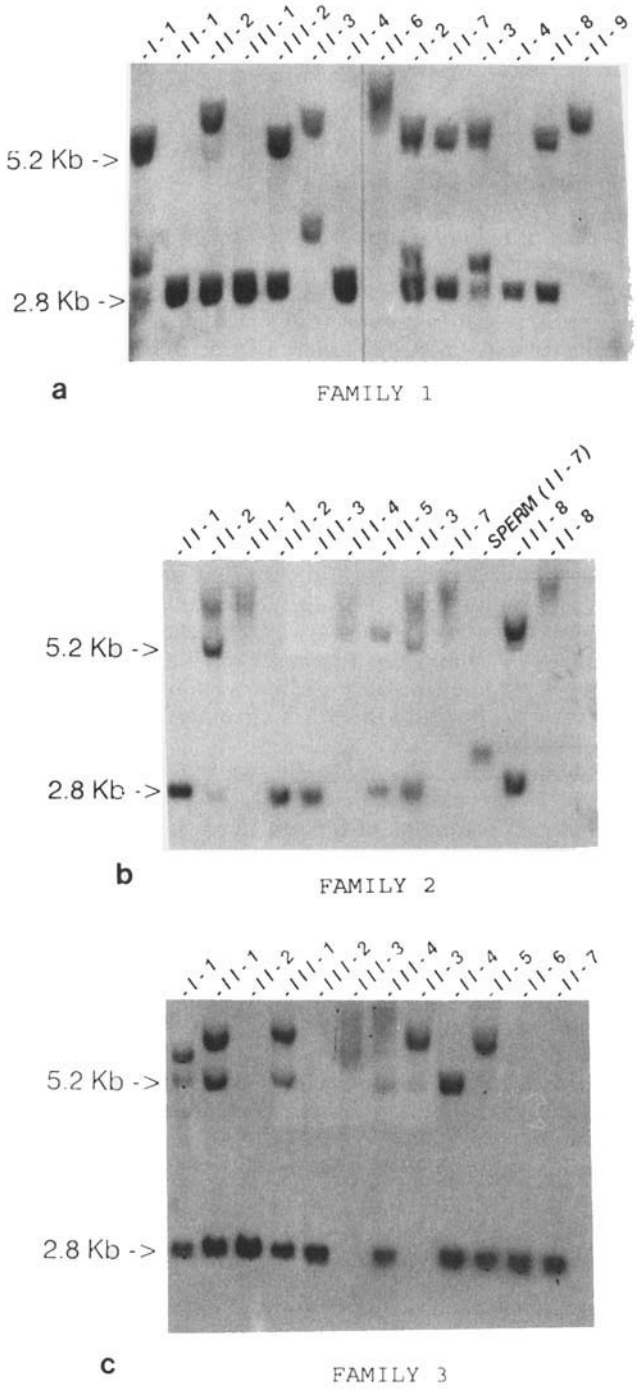


Fig. 2. Southern blot analysis of families 1 (**a**), 2 (**b**), and 3 (**c**) after HindIII + NotI digestion and hybridization with the probe OxE20. Normal unmethylated HindIII – NotI fragment and normal methylated HindIII fragment are approximately 2.8 and 5.2 Kb, respectively.

## RESULTS

Families 1 and 2 were reported as families 5 and 6, respectively, in Mulley et al. [1995]. Improved techniques and longer exposure times have allowed more accurate measurements of increments and methylation status, which in some cases differ from those reported previously.

### Family 1 (Figs. 1a, 2a)

This family was referred to confirm the FRAXA mutation because a high degree of fragility was observed in the propositus (II-6) when cultured lymphocytes were induced for FRAXA expression.

Because none of the studied relatives showed increments at the FRAXA locus with the StB12.3 probe (data not shown), a FRAXE or FRAXF fragile site was suspected, without the association of mental retardation [Sutherland and Baker, 1992].

To assess the carrier status of the apparently normal nonexpressing pregnant cousin (II-8), linkage analysis with Xq27-28 probes 1A1 and U6.2 was performed. This analysis established that mother (I-1) of the propositus, the apparently normal brother and sisters (II-3, II-2, and II-5), and the aunts (I-2 and I-3) were carriers of the mutated X, but the pregnant cousin was not.

After the cloning of the FRAXE locus [Knight et al., 1993], we reinvestigated this family. Figure 2a shows increments and methylation status at the FRAXE locus with probe OxE20. The propositus (II-6), a slightly retarded boy (IQ of 66) with 43% of fragility expression, showed a broad, fully methylated band over 7.5 Kb ($\Delta$ = 2.3) and a slight unmethylated band of 3.2 kb ($\Delta$ = 0.4).

All the females in generation I were carriers of short extra fragments (200–300 pb) that were either methylated or unmethylated over the inactive or active X chromosome, respectively. These fragments were incremented and methylated when passed to their sons and daugthers. The apparently normal sister (II-2) had 2 bands, 1 at 2.8 Kb and another weak band at 5.2, plus an extra methylated band of 6.5 kb ($\Delta$ = 1.3), which was absent in her children (III-1 and III-2). The normal sister (II-5) had 2 bands at the normal range size, plus a methylated fragment at 6.7 kb ($\Delta$ = 1.5; not shown in Fig. 2B). The normal carrier brother (II-3) surprisingly showed a mosaic pattern with 2 fragments of 3.4 Kb and 5.8 Kb ($\Delta$ = 0.6) over the unmethylated and methylated normal fragments. Individuals II-1, II-4, and II-7 had normal-sized fragments.

Individual II-9 is a moderately retarded boy (IQ of 49), who did not express fragility, with a nearly fully methylated increment of 0.7 Kb at the FRAXE locus. His sister (II-8) is not a carrier, as we demonstrated previously.

### Family 2 (Figs. 1b, 2b)

Individual III-1 was ascertained 10 years ago in a special FRAXA screening at school because of familial nonspecific mental retardation; curiously, this family was used repeatedly for updating techniques for FRAXA cytogenetics expression. The index case and several relatives showed fragility expression associated with mental impairment in males and females.

This family was reinvestigated for FRAXA mutation when the woman (II-3) was pregnant. Molecular study with the StB12.3 probe did not demonstrate an alteration at the FRAXA locus (data not shown). Amniocytes

showed a high degree of fragility (25%) in a male karyotype, and the FRAXE locus was investigated in the family with the OxE20 probe (Fig. 2B).

The propositus (III-1; IQ of 28) had a fully methylated smear between 6.2 and 8 kb (Δ = 1–2.8), whereas his slightly retarded brother (III-4; IQ of 62) showed a mosaic pattern with 2 methylated bands of 5.6 kb (Δ = 0.4) and 7.2 kb (Δ = 2) plus an extra unmethylated weak band of 3.1 kb (Δ = 0.3). The mother (II-2) and the pregnant aunt (II-3) developed normal methylated and unmethylated bands plus a broad methylated one between 5.6 and 8 Kb (Δ = 0.6–2.8). When confronted with these results, the limited available experience, and the variable phenotype showed in this family, this woman decided to continue the pregnancy.

The uncle (II-7) had a methylated smear between 5.8 and 8 kb (Δ = 0.6–2.8). This man has married twice and has had several unskilled jobs. We were able to analyze his sperm DNA (Fig. 2B, lane 10), which showed an unmethylated band of 3.1 kb (Δ = 0.3). This man has a fra-X-expressing, mildly retarded daughter (III-8; IQ of 57) who needs special schooling. This girl presents the normal female pattern plus an incremented band of 400 kb that is nearly fully methylated. The uncle (II-8) shows a methylated smear between 6.8 and 8 kb (Δ = 1.6–2.8). The mental status of this man was not well known. At present, we know that he is mentally affected and receives social help.

Although the grandmother did not express FRAXE, she is an obligate carrier as her sons (II-7 and II-8) are carrier males.

Individual III-5 has a 5.2-kb delayed band, which is considered a migration artifact because it was not observed in other Southern blots. Furthermore, PCR amplification of these CGG FRAXE triplets showed 2 normal alleles of 18 and 21 repeats of maternal and paternal origin, respectively (data not shown).

The remaining relatives were inaccessible for molecular analysis.

TABLE I. Similarities (+) and Differences (−) Between Propositi in Families 2 (2-III-1 and 2-III-4) and 3 (3-II-3) and Signs Typical of Fragile X Syndrome

| Principal anomalies | 2-III-1 | 2-III-4 | 3-II-3 | FRAXA |
|---|---|---|---|---|
| Mental retardation | + | +/− | + | + |
| Hyperactivity | + | − | − | + |
| Brachycephaly | + | + | + | − |
| Narrow forehead | + | + | + | − |
| Telecanthus | +/− | + | − | − |
| Broad nose | + | + | − | − |
| Full lips | + | + | − | − |
| High arched palate | + | + | + | −/+ |
| Broad neck | + | + | − | − |
| Big and prominent ears | − | − | − | + |
| Pectus excavatum | + | + | + | −/+ |
| Wide hands | + | − | − | + |
| Hyper-extensibility of joints | − | − | − | + |
| Palmar creases | − | − | − | + |
| Macrorchidism | − | − | + | + |

### Family 3 (Figs. 1c, 2c)

The consultant (II-1) was a 37-year-old pregnant woman, who had a mentally retarded brother (II-3); both showed chromosomal Xqter fragility. Analysis of the FRAXA region with the StB12.3 probe showed a normal result, whereas that with the OxE.20 probe demonstrated unstable increments in the FRAXE locus.

Cytogenetic prenatal diagnosis (III-4) was performed at 16 weeks and showed a female karyotype with 25% fragility in amniotics cells. Because of the lack of correspondence between fragility expression and mental handicap (mainly in carrier females), this woman decided to continue the pregnancy.

Figure 2C shows the transmission of the unstable FRAXE element in this family. The maternal grandmother (I-1) showed an extra methylated fragment of 6 Kb (Δ = 0.8) and the 2 normal 2.8- and 5.2-Kb fragments. Cytogenetic study of this woman was not conclusive, with fragility in both X chromosomes in 1 of 25 cells studied.

The mutated X chromosome was inherited as incremented by the affected propositus (II-3), who showed a methylated fragment of 6.8 Kb (Δ = 1.6), and by the normal carrier women (II-1 and II-5), who showed extra fully methylated fragments of 6.5 and 6.6 Kb, respectively (Δ = 1.3 and 1.4).

In the next generation, the FRAXE element was further incremented, showing more unstable behavior. III-1 is an apparently normal woman with an extra fully methylated fragment at 7 Kb (Δ = 1.8), and her brother (III-3), an 11-year-old, apparently normal, boy, showed a broad methylated smear between 6 and 9.5 kb (Δ = 0.8–4.3), whereas III-4 (previously tested in amniocytes) showed an extra methylated band of 7–9.5 kb (Δ = 1.8–4.3 ). She is too young to be psychologically evaluated, although she seems normal by pediatric evaluation.

In this family, there was agreement between molecular data and cytogenetic expression but not with mental impairment.

Although some affected males in these 3 families showed mild craniofacial anomalies, there was no consistent clinical phenotype. Table I shows similarities and differences between propositi from families 2 and 3 and signs typical of fragile X syndrome patients. It is remarkable that individual III-1 of family 2 showed hyperactivity and that the index case (II-3) of family 3 had macroorchidism.

## DISCUSSION

In contrast with the Martin-Bell phenotype shown by FRAXA males, fragility at the FRAXE locus has been associated with mild mental impairment without any other specific clinical phenotype [Knight et al., 1993].

Mental status in FRAXE individuals is highly variable, and even if mild mental retardation is observed in most cases, several carrier males have been reported to be apparently normal. In 1 case, the normal carrier was a mosaic with small unmethylated and large methylated increments [individual 2 in family 2 reported by Knight et al., 1993]. In another case, the individual showed a mosaic pattern of methylation, with an incremented fragment of 1.1 Kb 50% methylated [individual IV-2 in family 2 reported by Mulley et al., 1995]. Other individuals showed fully methylated large increments [Knight et al., 1994; Mulley et al., 1995]. All of them expressed FRAXE.

In family 1, II-3 showed a mosaic pattern for methylation with an incremented fragment of 600 bp 50% methylated. This individual was apparently normal by clinical impression, although an aggressive character was noted. His IQ was 101. Cytogenetic fragility was not tested.

Individual III-3 in family 3 showed a broad, fully methylated smear, and he expressed 16% of fragility. This carrier individual showed normal behavior without apparent mental impairment. In contrast, mosaic carrier males II-6 and II-9 from family 1 and III-4 from family 2 showed mild mental retardation.

In family 2, 2 carrier brothers showed a very different phenotype: III-1 was a hyperactive, severely retarded boy and III-4 merely showed poor performance at school and no hyperactivity. This boy demonstrated a mosaic pattern, with a 300-bp incremented unmethylated fragment plus a methylated smear.

This apparent dissociation between mental retardation, size increment, and methylation status at the FRAXE locus might be explained by tissue mosaicism, which showed different increments and methylation patterns in different tissues. The same has been observed in myotonic dystrophy [Ashizawa et al., 1993]. It is noteworthy that, in contrast with FRAXA, a vague correlation has been observed between expanded triplets at FRAXE and methylation status in the associated CpG island; carrier individuals with increments of 1.1 Kb [Mulley et al., 1995] or 600 bp [this study] that are 50% methylated, when combined with increments of 400 bp [Mulley et al., 1995] or 550 bp [Knight et al., 1994] that are fully methylated have been described.

FRAXE locus methylation may not as strictly associated with size at CGG triplets as that in FRAXA, and it is possible that normal carrier individuals with fully methylated increments in lymphocytes have a certain proportion of unmethylated aleles in the critical (i.e., neural) tissues.

Carrier females also showed a variable phenotype; however, this might be influenced by random X inactivation ocurring early in embryogenesis, which can skew the proportion of active and inactive X chromosomes.

In contrast with FRAXA, where nonpenetrant carrier males have only small unmethylated increments (premutation), the apparently unaffected FRAXE carrier males have a more complex pattern with small unmethylated increments or large methylated ones. In family 1, 3 women in generation I have small increments of 200–300 bp. All of them transmitted their alleles as incremented and partially or fully methylated fragments, which may reflect less stable behavior of small increments at this locus.

Increments and reductions of the CGG triplet have been observed in female transmission of FRAXE mutation. Initially, several transmissions of the FRAXE mutation from carrier males to their normal carrier daugthers, with reduction of the triplet number, were reported [Knight et al.,1993, 1994; Mulley et al., 1995]. This fact indicates a similarity to FRAXA in that only premutated alleles are present in the sperm of full mutation carrier males in peripheral lymphocytes [Reyners et al., 1993]. However, in 1 case, a mentally impaired female resulted with an incremented methylated fragment of 0.8 Kb [Hamel et al., 1994]. In family 2, the affected carrier male II-7, who showed a fully methylated smear from 0.4 to 2.8 Kb, trasmitted a fragment of 0.4 Kb, nearly fully methylated, to his slightly retarded daugther (III-8; IQ of 57). This girl, unlike the previously reported one, expressed fragility at Xqter.

We studied the elongated FRAXE fragment in sperm DNA from II-7 and observed an incremented fragment of 300 bp that was not methylated. Apparently, FRAXE mutation is similar to FRAXA, as males with somatic large methylated increments are carriers of small unmethylated ones in germinal cells. Although the observed biased inactivation in III-8 could be due to specific inactivation of the mutated X chromosome, this bias could be a consequence of the random X chromosome inactivation in females.

Although it seems likely that the FRAXE phenotype results from the loss of expression of a gene, the FRAXE mutation might affect FMR-1 expression [Knight et al., 1994], similar to the transcriptional inhibition reported at the IDS locus of FRAXA individuals [Clarke et al., 1992]. If so, similarity to the FRAXA phenotype is possible in some FRAXE carriers. In family 2, the index case (III-1) is a severely retarded male with hyperactive and repetitive behavior, which is frequently found in FRAXA-effected males. Moreover, the propositus in family 3 (II-3) showed macroorchidism at clinical evaluation. None of these traits has been reported previously in FRAXE males.

Aside from the heterogeneous pattern shown by FRAXE mutation, we emphasize the significant role that the familial environment might play on the phenotype developed by carrier individuals because stimulating training could significantly modulate the mild phenotype. All the families in the present study live under poor sociocultural conditions.

FRAXE mutation seems to be less frequent than FRAXA. We found 3 FRAXE families among 70 studied with fragile X expression, but because of the very mild phenotype shown in FRAXE carrier individuals, they might not come to our attention as frequently as FRAXA carriers.

Screening children with learning difficulties and others to assess the frequency of FRAXE mutation and the clinical variety of the disease should be done.

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